

ADMET & DMPK 1(2) (2013) 6-16; doi: 10.5599/admet.1.2.3



Open Access : ISSN : 1848-7718

<http://www.pub.iapchem.org/ojs/index.php/admet/index>

Original scientific paper

Physico-chemical Profiling of the ACE-inhibitor Lisinopril: Acid-base Properties

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Received: January 7th, 2013; Revised: February 1st, 2013; Published: February 15th, 2013

Abstract

The acid-base chemistry of a tetraprotic, ampholyte ACE inhibitor, lisinopril, was studied by different methods. Potentiometry in aqueous medium and a co-solvent technique in methanol-water mixtures as well as ¹H NMR-pH titration were applied for the highly precise measurement of protonation macroconstants. The log K values of lisinopril (at 25.0°C and 0.15 M ionic strength) were found: log K₁ = 10.75 ± 0.01, log K₂ = 7.13 ± 0.01, log K₃ = 3.13 ± 0.01, log K₄ = 1.63 ± 0.01, calculated as an average of the best two values obtained by independent methods. NMR-pH titration was used to assign the constants to the functional groups and for the examination of site-specific, submolecular basicities of the molecule (determination of protonation microconstants). In the first two well-separated protonation steps, the macro- and microconstants were identical and assigned to the primary amino group (log K₁ = log k^A) and to the secondary amine basicity (log K₂ = log k^B_A), respectively. The two carboxylates exhibited overlapping protonation characterised first by microconstants (log k^D_{AB} = 2.15, log k^C_{AB} = 3.10), revealing that the carboxylate on the proline ring has nine times greater intrinsic basicity than the carboxylate on the side chain. The distribution of protonation species (Lis²⁻; HLis⁻; H₂Lis; H₃Lis⁺; H₄Lis²⁺) and microspecies (ABC; ABD) as a function of pH was calculated and used to interpret the pharmacokinetic and pharmacodynamic properties of lisinopril.

Keywords

proton speciation; logK; potentiometry; NMR-pH titration

Introduction

Angiotensin converting enzyme (ACE) inhibitors serve as fundamental medicines in the treatment of hypertension, one of the most prevalent chronic diseases nowadays. Out of the numerous currently available ACE-inhibitors, lisinopril – (2S)-1-[(2S)-6-amino-2[[[(1S)-1-carboxy-3-phenylpropyl]amino]-hexanoyl]-pyrrole-2-carboxylic acid (Fig. 1) – belongs to the proline-containing structures.

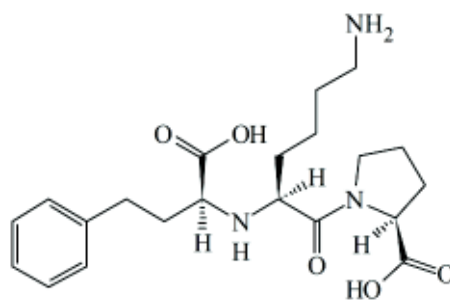


Figure 1. Chemical structure of the ACE inhibitor lisinopril

Due to its safe and effective properties, lisinopril is widely used in the therapy of essential hypertension, symptomatic and asymptomatic left ventricular systolic dysfunction, post-myocardial infarction, renal failure and diabetic nephropathy [1]. Lisinopril is administered orally usually in a daily dose of 2.5-40 mg. Its oral bioavailability is between 25-30 % [2]. Absorption from the gastrointestinal tract does not depend on nutrition. Six hours are needed to reach the maximum plasma concentration. It hardly binds to plasma proteins. Lisinopril is not metabolised, and the absorbed drug is primarily excreted invariably in the urine [3].

A vast literature describes the pharmacology, mechanism of action, pharmacokinetics and analytics of lisinopril [3-7]. However, surprisingly little information is available on its physico-chemical properties. Lisinopril is a polyfunctional, ampholyte molecule, containing two basic and two acidic moieties. Various data have been published on its acid-base chemistry. The dissociation constants (pK_a values) were reported by Bennion *et al.* [8] (1.68, 3.29, 7.01, 11.12) and Ip *et al.* [9] (2.5, 4.0, 6.7, 10.1), without an indication of the experimental error and with no assignment of basicities to moieties. Gonzalez *et al.* [10] assigned the measured pK_a values (1.4 ± 0.1 , 3.00 ± 0.01 , 7.10 ± 0.01 , 10.78 ± 0.01) to the functional groups, but only on the basis of chemical evidence. These three datasets of pK_a values are significantly different, particularly the lowest and highest constants, which remain uncertain. The difference between them exceeds one order of magnitude.

The acid/base character determines the charge of a molecule in solution at a particular pH (characterised by the dissociation/protonation constant, $pK_a/\log K$). Further on in this paper, we use the $\log K$ term and consider the ionisation process as an association with a proton in all acid-base equilibria. This information is important in the estimation of ADME (absorption, distribution, metabolism, excretion) parameters and the interpretation of pharmacokinetic (PK) properties. $\log K$ values can be used to better understand the binding mechanism in therapeutic events and also for optimisation of chemical reactions and analytical methods.

Several analytical methods have been applied for the determination of lisinopril in biological samples and pharmaceutical preparations such as alkalimetry [11], spectrophotometry [12], high performance liquid chromatography (HPLC) [13], high performance thin-layer chromatography (HPTLC) [14], and capillary electrophoresis (CE) [15]. Obviously, upon the application of these methods, exact knowledge of acid-base chemistry is essential (for pharmacopoeial methods) or at least favourable (for chromatographic techniques and CE).

The acidity/basicity of monovalent compounds can be quantified in terms of macroscopic $\log K$ parameters (macroconstants). For multiprotic compounds, macroconstants characterise the basicity of the molecule as a whole. They refer to the stoichiometric composition of the species, but they fail to provide information on specific proton-binding sites. Site-specific, submolecular basicities can be obtained when

microconstants are determined. Microconstants measure the proton affinity of individual groups, while the protonation states of all other sites are definite in the molecule [16]. The macroscopic and microscopic basicities provide authentic information on propensities of intermolecular interactions both in pharmacokinetic (PK) and pharmacodynamic (PD) stages [17].

As a part of our studies on the physico-chemical properties of ACE inhibitors, we investigated the acid-base chemistry of lisinopril. We characterised both the overall and the group-specific basicities of lisinopril. Validated potentiometric methods and ^1H NMR spectroscopy resulted in more precise macroconstants than in previous investigations. Here, we first studied the site-specific (microscopic) protonation equilibria of lisinopril considering the overlapping protonation of carboxylates. The results were used to interpret the PK/PD properties of the compound.

Experimental

Materials and instrumentation

Lisinopril dihydrate was generously supplied by Gedeon Richter Ltd. (Budapest, Hungary) and used without further purification. Distilled water was of pharmacopoeial grade [11] and all other reagents of analytical grade were purchased from commercial suppliers.

Potentiometric titrations were carried out on a GLpKa automated pK_a and $\log P$ analyser (Sirius Analytical Instr. Ltd. Forest Row, UK).

NMR measurements were carried out on a 600 MHz Varian Inova spectrometer (Palo Alto, CA), equipped with a broadband inverse detection pulse field gradient probehead.

Potentiometric logK determination

Electrode calibration

The four-parameter procedure was used for electrode standardisation in both aqueous and semi-aqueous media [18]. HCl solutions of known concentration, containing 0–45.68 wt% methanol, were titrated with standardised KOH at 25.0 ± 0.1 °C, at 0.15 M ionic strength using KCl, under an N_2 atmosphere, in the pH interval of 1.8–12.2. The operational pH reading was related to $\text{p}_\text{c}\text{H}$ values by the standard multiparametric equation:

$$\text{pH} = a + S\text{p}_\text{c}\text{H} + j_\text{H}[\text{H}^+] + j_\text{OH}K_\text{w}/[\text{H}^+] \quad (1)$$

where a corresponds to the negative logarithm of the activity coefficient of $[\text{H}^+]$ at working temperature and ionic strength and S is the ratio between the electrode slope and the Nernst slope. The j_H and j_OH terms correct the electrode junction effects at low and high pH, respectively.

The parameters were determined by a weighted non-linear least squares procedure (Refinement ProTM 2.2 software - Sirius Analytical Instr. Ltd. Forest Row, UK).

Titration in aqueous medium

Six millilitres of 1.97–2.10 mM aqueous solutions of the sample were preacidified to pH 1.8 with 0.5 M HCl and then titrated alkalimetrically to pH 12. The titrations were carried out at 25.0 ± 0.1 °C, at 0.15 M ionic strength using KCl under an N_2 atmosphere. Three separate titrations were performed. The initial

estimates of $\log K$ values were obtained from Bjerrum difference plots (\bar{n} vs. pH, when \bar{n} is the average number of bound protons) and were refined by a weighted non-linear least squares procedure using Refinement Pro™ 2.2 software (Sirius Analytical Instr. Ltd. Forest Row, UK).

Titration in solvent mixtures

Three semi-aqueous solutions of lisinopril containing 14.21, 28.54 and 43.82 wt% methanol were titrated under the same experimental conditions as in aqueous medium. The apparent protonation constants ($\log_s K$) were calculated from the difference (Bjerrum) plot in a similar manner as the aqueous $\log K$ values. The Yasuda-Shedlovsky procedure was applied to estimate the aqueous $\log K$ values. The Yasuda-Shedlovsky extrapolation method is based on the linear relation between $\log_s K$ and the dielectric constant (ϵ) of a solvent mixture:

$$\log_s K + \log [\text{H}_2\text{O}] = a / \epsilon + b \quad (2)$$

where a is the slope, b is the intercept (fitting constant) of the linear equation, ϵ is the dielectric constant of the methanol-water mixture and $\log [\text{H}_2\text{O}]$ is the molar water concentration of the given solvent mixture. This method is a widely used procedure in co-solvent techniques [19,20].

¹H NMR titration with in situ pH monitoring

A single NMR sample solution of 0.6 ml was prepared containing 8 mM lisinopril, 140 mM NaCl (to ensure a constant ionic strength of 0.15 M) in a 9/1 H₂O/D₂O solvent mixture. This sample also contained 2 mM of the following pH indicator molecules: dichloroacetic acid, chloroacetic acid, acetic acid, imidazole, tris(hydroxymethyl)-aminomethane (TRIS) and trimethylamine hydrochloride (TMA) in order to determine the actual pH of the sample in each titration step, according to the electrodeless single tube NMR titration method [21]. As an internal chemical shift reference, 0.5 mM of sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) was applied. The ¹H NMR spectra were recorded at 25.0 ± 0.1 °C. The water signal was suppressed either by the double pulse field gradient spin echo (*dpfgse*) or the selective presaturation (*presat*) sequence. The *in situ* pH value was deduced from the chemical shift of the appropriate indicator by the following equation:

$$\text{pH} = \log K_{\text{Ind}} + \log \frac{\delta_{\text{Ind}}^{\text{obs}} - \delta_{\text{HInd}}}{\delta_{\text{Ind}} - \delta_{\text{Ind}}^{\text{obs}}} \quad (3)$$

where $\log K_{\text{Ind}}$ is the protonation constant of the indicator and δ_{Ind} , δ_{HInd} are the limiting chemical shifts of its non-protonated and protonated forms (determined in separate experiments) [22,23].

The NMR-pH datasets were fitted with the Opium computer program [24] to determine protonation macroconstants and chemical shift values specific to each macrospecies (*dH_iLis*).

Results and Discussion

Among the ACE inhibitor drugs, lisinopril has the most interesting and complex acid-base chemistry. There are four proton-binding sites in the molecule: two carboxylates as well as a primary and a secondary

amine group. The protonation processes of the two carboxylate groups are expected to be highly overlapping. For the investigation of the ionisation/protonation properties of the molecule, potentiometry in aqueous medium and in methanol-water mixtures as well as NMR-pH titration were applied.

Protonation macroconstants

The stepwise protonation equilibria (Fig. 2) of the whole molecule are characterised in terms of protonation macroconstants, $\log K$ values.

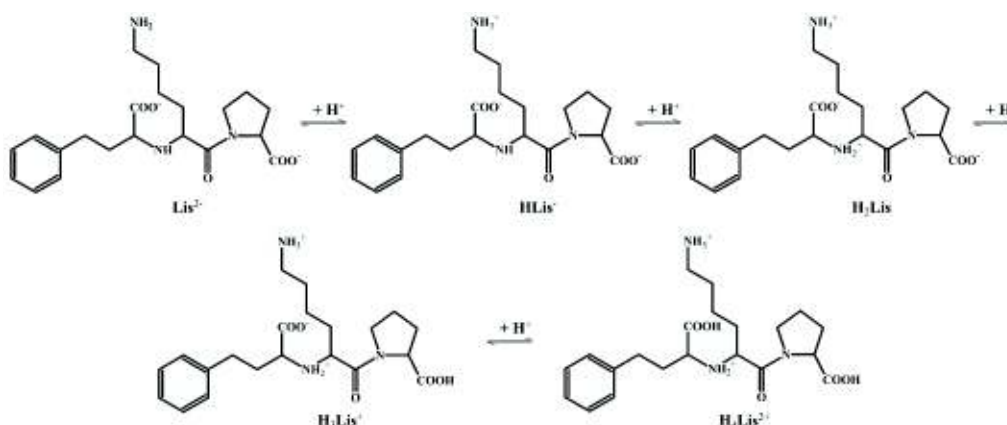


Figure 2. Stepwise protonation equilibria of lisinopril

Generally, potentiometry in aqueous medium is the method of choice for $\log K$ determination for molecules with solubility greater than 0.8 mM in the entire pH interval of interest. The solubility of lisinopril (0.22 M, [9]) allows the determination of macroconstants by the standard method. The four obtained $\log K$ values along with the standard deviations calculated from three parallel titrations (3 x 15 points) are listed in Table 1.

Since the $\log K_4$ value falls below to the lower applicability limit ($\log K < 2$) of pH-metric titration, we also measured the protonation macroconstants using the co-solvent method. The effect of methanol on the protonation constant is known to depend on the charge of the basic site. For acids, the apparent $\log_5 K$ value increases with the increasing weight percent of methanol, while a decrease is usually observed for bases [25]. Using this co-solvent method, we obtained a more reliable value for $\log K_4$ since the $\log_5 K_4$ values in methanol-water mixtures shifted up to the measurable pH range. At the same time, this co-solvent method allows for assigning $\log K$ values to the acidic and basic moieties of the molecule. According to the slopes of the regression lines on the Yasuda-Shedlovsky plot (Fig. 3), $\log K_1$ and $\log K_2$ characterise amine functions, while $\log K_3$ and $\log K_4$ the carboxylate groups of the molecule, respectively.

For the exact proton speciation of lisinopril, ^1H NMR-pH titrations were carried out using the most similar possible experimental conditions as in potentiometry. Fig. 4 shows the aliphatic part of one spectrum from the titration series.

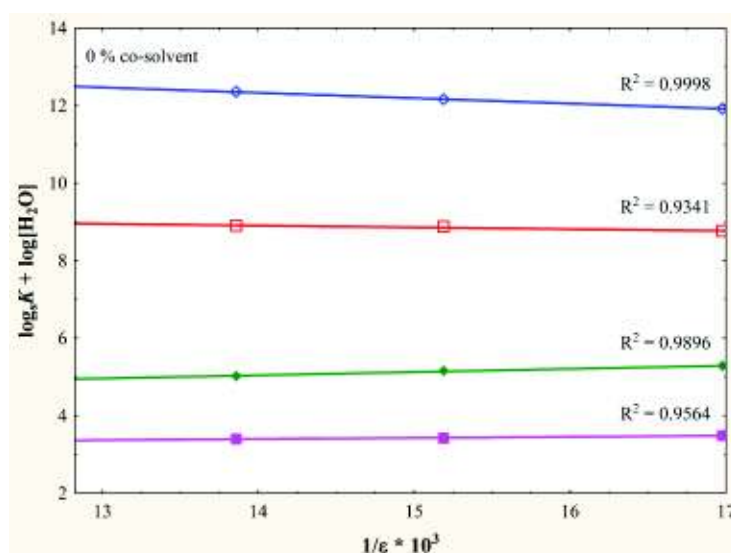


Figure 3. Yasuda-Shedlovsky plot where open diamonds denote $\log K_1$ (slope: -138.4, intercept: 14.271), open squares denote $\log K_2$ (slope: -44.6, intercept: 9.532), filled diamonds denote $\log K_3$ (slope: 82.6, intercept: 3.891) and filled squares denote $\log K_4$ (slope: 29.9, intercept: 2.976).

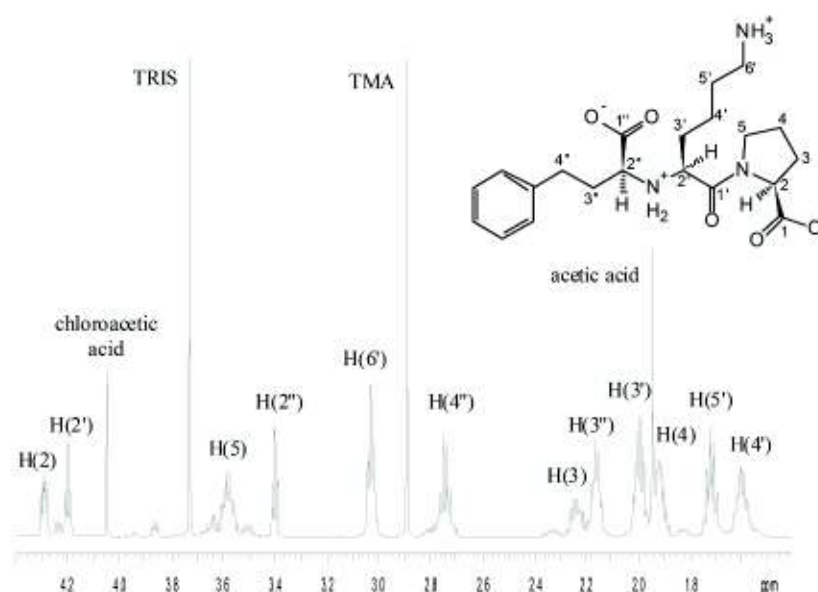


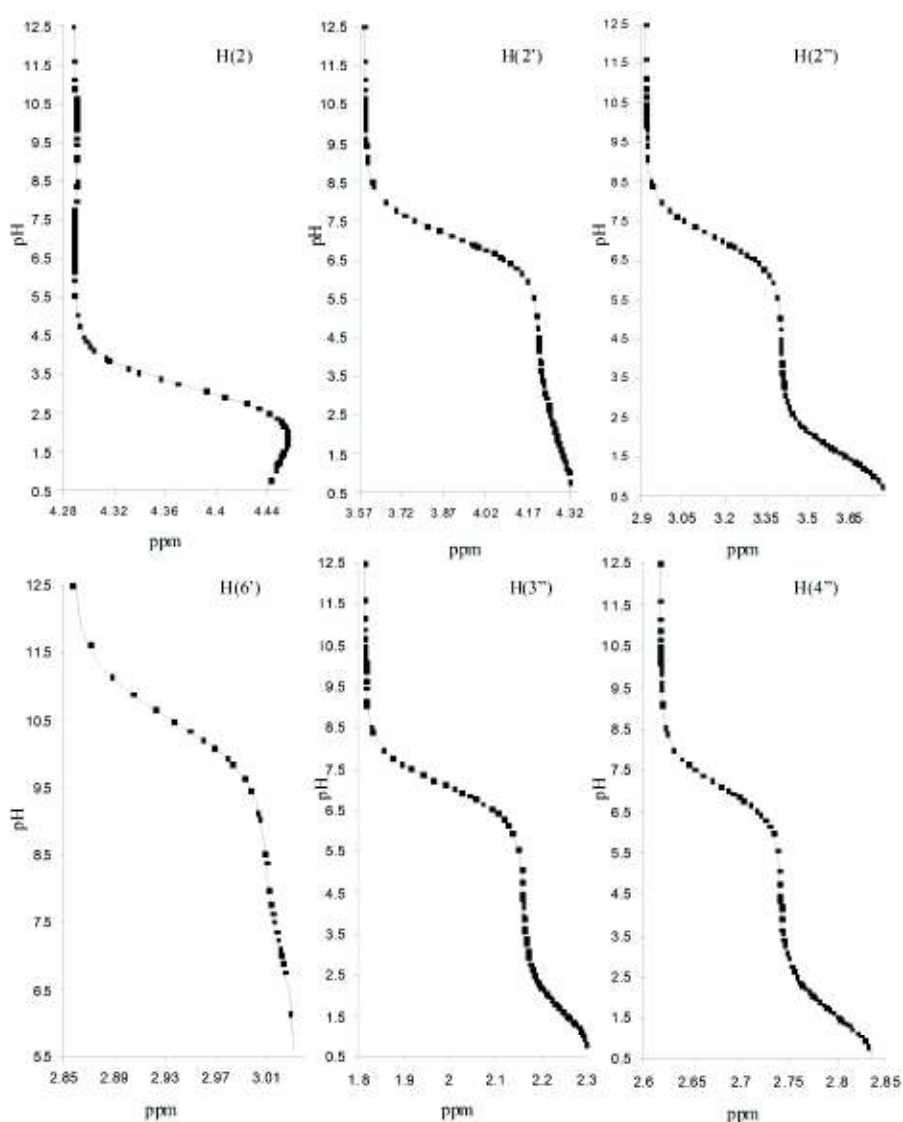
Figure 4. Representative ^1H NMR spectrum of lisinopril at pH 5.07 in 9/1 $\text{H}_2\text{O}/\text{D}_2\text{O}$. The assignment is based on conventional 2D NMR techniques.

At certain pH values, the assignment of overlapping multiplets to methylene and methine protons was verified by TOCSY and HSQC experiments. In Fig. 5, the chemical shifts of the methine protons H(2), H(2') and H(2'') along with the methylenes H(6'), H(3'') and H(4'') are depicted as functions of pH. These six datasets were fitted simultaneously to the tetraprotic macroscopic model function described elsewhere [26]. The obtained macroconstants are shown in Table 1.

Table 1. Protonation macroconstants measured by different methods.

Method	Protonation macroconstants			
	$\log K_1 \pm \text{SD}$	$\log K_2 \pm \text{SD}$	$\log K_3 \pm \text{SD}$	$\log K_4 \pm \text{SD}$
potentiometry in aqueous medium	10.74 ± 0.01	7.14 ± 0.01	3.10 ± 0.01	1.54 ± 0.05
potentiometry in solvent mixtures	10.75 ± 0.01	7.22 ± 0.03	3.21 ± 0.02	1.62 ± 0.01
NMR-pH titration	10.53 ± 0.03	7.12 ± 0.01	3.15 ± 0.01	1.63 ± 0.01
average of the best two values*	10.75 ± 0.01	7.13 ± 0.01	3.13 ± 0.01	1.63 ± 0.01

* see details in text

**Figure 5.** NMR-pH titration curves with computer fitted solid line

The macroconstants (Table 1) obtained by different methods show generally good agreement. The average deviation between the $\log K$ values was 0.09 units. The ca. 0.2 unit discrepancy in $\log K_1$ between data acquired by the NMR and potentiometric methods may arise from the greater ambiguity of *in situ* pH values. Specifically, trimethylamine has a $\log K_{\text{Ind}} = 9.90$ [23] and thus can monitor pH values higher than

10.9 with a lower precision (see the error estimations in [21] for details). In contrast, the $\log K_4 = 1.63$ value by NMR can be considered as more reliable than data obtained by potentiometry in aqueous medium, because below pH 2, the acidity error of the electrode is considerable, while here the NMR-pH titration as described above is an electrodeless technique. This constant could be determined with excellent precision from the extrapolation of semi-aqueous $\log_5 K$ values due to the reasons mentioned above.

However, we think it reasonable to make an average of the best two experimentally measured $\log K$ values based on the following principle. We omit the $\log K$ value with the highest uncertainty (the highest SD value). The calculated, most reliable protonation macroconstants are indicated in the last row of Table 1. Comparing the obtained $\log K$ values with those reported in [9], the difference was greater than 0.4 log units in all $\log K$ values. Both the $\log K_1$ determined by Benion et al. [8] and the $\log K_4$ reported by Gonzalez *et al.* [10] differ remarkably from our data. This is not surprising because those constants were deduced from conventional pH-potentiometry in aqueous medium, while the methods used in our study (potentiometry in methanol-water mixtures, ^1H NMR-titration) give more reliable protonation constants at pH extremes.

Protonation microconstants

Nevertheless, macroscopic constants could not directly be assigned to functional groups, since the difference in their logarithms did not exceed 3. The microscopic protonation scheme in Fig. 6 should be considered for lisinopril instead, where the superscript on microconstant k indicates the group protonating in the equilibrium in question, whereas the subscript (if any) refers to already protonated group(s).

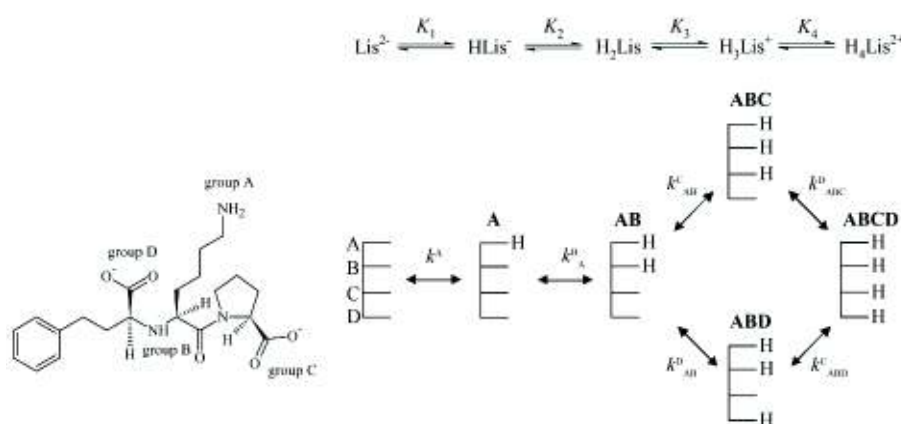


Figure 6. Site-specific (microscopic) protonation scheme of lisinopril

In the first two well-separated protonation steps, the macro- and microconstants are identical. Since the first protonation is reported most sensitively by H(6'), this protonation step can be assigned to the primary amino group ($\log K_1 = \log k^A$). Similarly, $\log K_2 (= \log k^B_A)$ accounts for the secondary amine basicity.

In the case of lisinopril, the carboxylates denoted by C and D exhibit overlapping protonation, so selective monitoring of at least one of them is a prerequisite for microconstant determination. According to the normalised NMR titration curves, H(3'') and H(4'') can be assumed as selective sensors of D carboxylate protonation. Thus, the experimental chemical shift profile of H(3'') was fitted to the following model equation:

$$\delta^{\text{obs}} = \delta_{H_2\text{Lis}} + (\delta_{H_4\text{Lis}^{2+}} - \delta_{H_2\text{Lis}}) \cdot \frac{k_{AB}^D[H^+] + K_3K_4[H^+]^2}{1 + K_3[H^+] + K_3K_4[H^+]^2} \quad (4)$$

The microscopic evaluation resulted in $\log k_{AB}^D = 2.15 \pm 0.05$ ($n = 2$) which, in turn could be converted to the remaining three microconstants, using the following Hessian constraints:

$$k_{AB}^D + k_{AB}^C = K_3 \quad (5)$$

$$k_{AB}^D k_{ABD}^C = k_{AB}^C k_{ABC}^D = K_3 K_4 \quad (6)$$

We obtained $\log k_{ABD}^C = 2.63$, $\log k_{AB}^C = 3.10$ and $\log k_{ABC}^D = 1.68$ (with estimated uncertainties of 0.05).

The main pathway of protonation includes the ABC microspecies, while its minor protonation isomer (ABD) has nine times lower abundance at all pH values. In other words, the carboxylate on the proline ring exhibits nine times greater intrinsic basicity than the carboxylate on the side chain with an adjacent, electron-attracting protonated secondary amine ($-\text{NH}_2^+$) group. The mutual basicity-modifying effect of these moieties is quantified by the interactivity parameter, defined as the difference in the corresponding microconstants: $\text{p}E_{\text{CD}} = 2.15 - 1.68 = 3.10 - 2.63 = 0.47$. This $\text{p}E$ value suggests an interaction exceeding the random, Coulombic value of 0.2-0.3 [27]; presumably, these sites communicate through space with a simultaneous change in their geometric positions. A conformational change at $\text{pH} < 4.5$ is also substantiated by the changing multiplet patterns in the NMR spectra, but a detailed analysis of vicinal $^3J_{\text{HH}}$ values holding conformational information is beyond the scope of this article.

Distribution of protonation species

Using $\log K$ and $\log k$ values, the percentage of various protonated species can be calculated for any arbitrary pH value. The distribution ratios of each species as a function of pH are shown in Fig. 7.

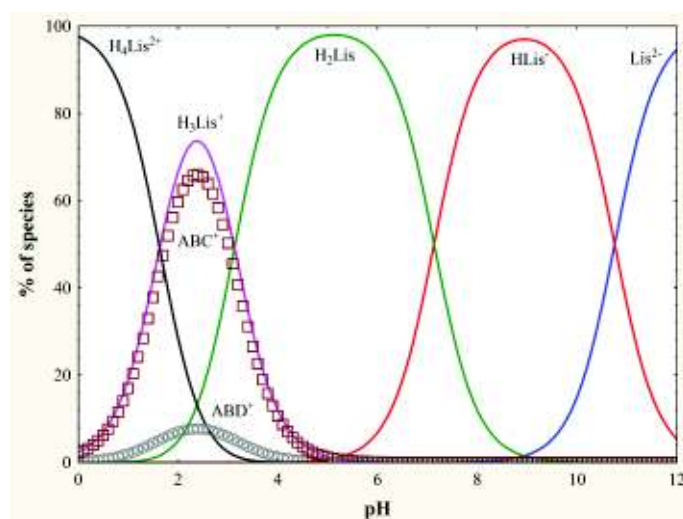


Figure 7. Distribution curves of the macrospecies ($H_4\text{Lis}$) and microspecies (ABC and ABD) of lisinopril

Table 2 summarises the relative concentration of lisinopril protonation species at the most relevant pH values in the body. In the stomach, the monocation (H_3Lis^+) and the dication ($\text{H}_4\text{Lis}^{2+}$) forms are present at about equal levels. In the gastrointestinal tract, the double protonated H_2Lis form predominates, while at the pH of plasma, the monoanion (HLis^-) is the dominant species.

Table 2. The percentage concentration of the species in the stomach (pH 1.5), gastrointestinal tract (jejunum pH 6.5) and plasma (pH 7.4).

Form of the species	Percentage concentration		
	stomach pH 1.5	GI tract (jejunum) pH 6.5	plasma pH 7.4
Lis^{2-}	0.00	0.00	0.03
HLis^-	0.00	18.98	65.04
H_2Lis	0.99	80.98	34.93
H_3Lis^+	42.15	0.03	0.00
$\text{H}_4\text{Lis}^{2+}$	56.86	0.00	0.00

Conclusions

In this study, the acid-base chemistry of tetraprotic lisinopril was characterised by protonation macro- and microconstants. The highly precise log K values obtained by two independent analytical methods provided a better interpretation of the PK/PD properties. As Fig. 7 indicates, under the pH conditions of the GI tract – the most likely site of oral absorption – lisinopril is predominantly present in the H_2Lis form. Although the net charge of this species is zero, the molecule exists in solution as a double zwitterion: the two amine functions are protonated while the two carboxylates are not. This structure represents a highly polar dipole that is unfavourable for passive transport through lipid membranes, which explains the low bioavailability of the molecule. Recently, the intestinal dipeptide transporter system (DTS) has been reported to be involved in the active transport mechanism of ACE inhibitors [28].

At the pH of the plasma, the monoanion (HLis^-) is the dominant form (more than 60%), which favours receptor binding. As is known, therapeutically useful ACE inhibitors exhibit three-point binding to the Zn ion-containing carboxypeptidase ACE enzyme [2]. The proline C_2 -carboxylate of lisinopril binds to the positively charged Arg^{146} , while the other carboxylate in the side chain participates in complex formation with the Zn ion. The third binding site is represented by Ser-OH where the $\text{C}_1=\text{O}$ group forms an H-bond. The species distribution revealed in this study explains the good receptor binding of lisinopril.

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